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Interactions of olopatadine and selected antihistamines with model and natural membranes

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Abstract *Objective:* Olopatadine, an effective topical ocular human conjunctival mast cell stabilizer/antihistaminic antiallergic drug, was evaluated and compared to selected classical antihistamines for their interaction with model and natural membranes to ascertain potential functional consequences of such interactions. *Methods:* The model membranes examined consisted of the argon-buffer interface and monomolecular films of 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) at the argon-buffer interface. Interactions with the model membranes were detected as changes in surface tension, i.e., surface pressure. Functional consequences of these interactions were assessed with natural membranes by 6-carboxyfluorescein leakage, hemoglobin release, lactate dehydrogenase release, and histamine release from appropriate cell types. *Results:* Measurements at the argon-buffer interface revealed intrinsic surface activity for all agents that ranged from highly surface-active to weakly surface-active in the order of: desloratadine > clemastine > azelastine \equiv ketotifen > diphenhydramine > pyrilamine > emedastine > epinastine \geq olopatadine. This order of amphipathic behavior was confirmed for most of the compounds by estimates of their dissociation constants ($K_{d,L}$) determined from interactions with SOPC monolayers adjusted to a surface pressure approximating that of natural membranes. Epinastine was the only antihistamine that showed a disproportionately greater increase in surface activity toward SOPC in monolayer when compared to other antihistamines. Dissociation constants could not be established for olopatadine because of its low affinity for both the argon-buffer inter-

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face and the SOPC monolayer. Functional consequences of these interactions were assessed with natural membranes by 6-carboxyfluorescein leakage (erythrocyte ghosts), hemoglobin release (erythrocytes), lactate dehydrogenase release (conjunctival mast cells, corneal epithelial cells), and histamine release (conjunctival mast cells). Aside from olopatadine and emedastine, all antihistamines promoted a concentration-dependent leakage of hemoglobin from intact erythrocytes. The concentration of drug required to cause half-maximal hemoglobin release (H_{50}) from erythrocytes correlated linearly ($r = 0.98$) with the SOPC dissociation constants ($K_{d,L}$) estimated for the different antihistaminic agents interacting with SOPC monolayers. A similarly high correlation ($r = 0.85$) emerged from a plot with a slope approaching unity that related drug concentrations required for half-maximal hemoglobin leakage from erythrocytes to threshold doses of drug that caused histamine release from human conjunctival mast cells. Olopatadine was the only agent that did not promote membrane perturbation as monitored by either hemoglobin release from intact erythrocytes, LDH release from human conjunctival mast cells, or 6-carboxyfluorescein release from erythrocyte ghosts. Assessment of the lytic potential of marketed concentrations of ketotifen (0.025%), azelastine (0.05%), and epinastine (0.05%) revealed significant membrane perturbation of human conjunctival mast cells and, importantly, human corneal epithelial cells as indexed by LDH release. This was in contrast to marketed concentrations of olopatadine (0.1%) which maintained normal mast cell and corneal epithelial cell membrane function. **Conclusions:** Combined, these results support the notion that the disruption of natural cell membranes by surface-active antihistamines occurs not through a receptor-mediated process, but is the consequence of a direct interaction of these agents with the cell membrane. This is corroborated by surface pressure-concentration isotherms for adsorption of five different antihistaminic agents to SOPC monolayers where 50% lysis occurred at a surface pressure of 42.9 ± 1.1 mN/m. Olopatadine appears to be unique among the agents tested by demonstrating low intrinsic surface activity, thus limiting its interaction with natural membranes. At concentrations of about half-maximal compound solubility (i.e., 5.0mM or a 0.19% drug solution), olopatadine generated SOPC monolayer surface pressures (i.e., 39.82 ± 0.10 mN/m) that were below those that promoted membrane perturbation and onset of hemoglobin leakage. Olopatadine's restricted interaction with membrane phospholipids limits the degree of membrane perturbation and release of intracellular constituents, including histamine, LDH, and hemoglobin, which is believed to contribute to olopatadine's topical ocular comfort and patient acceptance.

Key words Antihistamine; cytotoxicity; membrane effects; human conjunctival mast cells; olopatadine

Introduction It has been known since the 1960s that antihistamines both inhibit mediator release and stimulate histamine release from mast cells.¹ The mediator-releasing potencies and the mast cell-stabilizing potencies of the antihistamines are not related to their histamine H_1

receptor-binding affinities.^{3,3} These effects on mast cells may be due to the lipophilic nature and/or the local anesthetic nature of the drug structures on biological membranes.^{4,5} Insertion of lipophilic drugs into cell membranes can eventually cause membrane disruption and drug-induced release of pro-inflammatory mediators. Local anesthetics have been reported to bind to most cell membranes and expand and disrupt these membranes, leading to histamine release.⁶ The tendency of molecules to partition into biological membranes is called surface activity, a thermodynamic quantity that can be measured as the ability to lower the interfacial tension at the air/water or lipid/water interface. Surface activity can regulate drug biological activity⁷ as well as the tendency of drugs to first stabilize and then permeabilize biological membranes.⁸

Olopatadine is an antiallergic drug that possesses human conjunctival mast cell-stabilizing activity and H₁ histamine receptor antagonism.^{9,10} Interestingly, the compound does not stimulate histamine liberation from human conjunctival mast cells at concentrations at least 10-fold higher than the clinically effective concentration.^{11,12} Examination of olopatadine's ability to interact with numerous biologically relevant receptors indicates that the compound does not have significant affinity for β -receptors or various calcium channels.¹⁰ Olopatadine lacks local anesthetic activity at concentrations as high as 2.0% (w/v).¹³ Concentrations ranging from 100 nM to 100 μ M have no effect on mast cell cAMP content, indicating a lack of effect on phosphodiesterase.¹⁴ These data suggest that olopatadine has a mechanism of conjunctival mast cell stabilization that is distinct from that of a receptor-mediated process.

Previous studies have shown that antihistamines can be surface-active^{15,16} and can, as with mast cells, stabilize then permeabilize the erythrocyte membrane.¹⁷ To better understand why olopatadine does not cause histamine release from human conjunctival mast cells, we measured the surface activities of a group of antihistamines and compared them to their abilities to permeabilize biological membranes. Specifically, the surface activities of olopatadine and antihistamines were determined at both the argon/buffer interface (intrinsic surface activity) and monolayer films of a membrane-relevant phospholipid (lipid-dependent surface activity), i.e., 1-stearoyl-2-oleoyl-sn-glycero-3-phosphorylcholine (SOPC). Membrane permeability was characterized using three complementary in-vitro models. Intact bovine erythrocytes and ghosts derived from them are traditional models for permeability studies and were used to measure membrane leakage of both large (hemoglobin) and small (carboxyfluorescein) molecular weight components. A more directly relevant model, human conjunctival mast cells, was used to evaluate histamine release from intracellular granules and lactate dehydrogenase (LDH) release from the cytoplasmic compartment. A clinically relevant ocular model, LDH release from primary cultures of human corneal epithelial cells, was also evaluated. The results of these studies show that all antihistamines are surface-active, but olopatadine is less so than the others tested. Moreover, the compound's surface activity correlates strongly with the drug-induced permeability of cell membranes in all cell types examined, indicating a physical basis for olopatadine's lack of lytic effects.

Materials and methods

REAGENTS The antihistamines evaluated were either commercial products from Alcon Laboratories, Inc., or were synthesized at Alcon Research, Ltd. (Ft. Worth, TX, USA). Water was purified by reverse osmosis and carbon filtration, and sequential passage through an Elix 3 deionization system (Millipore) and a Milli Q UV Plus polishing system (Millipore). 6-Carboxyfluorescein (CF) was obtained from Molecular Probes (Eugene, OR, USA), SOPC from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), and Triton X-100 detergent from Sigma (St. Louis, MO, USA). OPTISOL[®] corneal preservation medium was from Bausch & Lomb (Rochester, NY, USA). FNC-coating mix was from BRFF (Ijamsville, MD, USA). Dispase II was a product from Roche Applied Science (Indianapolis, IN, USA). CYTOTOX 96[®] kits were obtained from Promega (Madison, WI, USA). Collagen I Cellware was from Becton Dickinson (Franklin Lakes, NJ, USA). KGM medium was from Biowhittaker (Walkersville, MD, USA). All other chemicals used were reagent-grade or highest purity available.

MEASUREMENT OF SURFACE ACTIVITY The technique, apparatus, and data analysis procedure for characterizing the interaction of solutes with SOPC monolayers were used as previously described by Brockmann et al.¹⁸ Briefly, an SOPC monolayer on an aqueous buffer subphase (0.1M NaCl, 10mM HEPES, pH 7.5) held at 24 °C and at constant area was exposed to increasing concentrations of the test solute. This was accomplished by continuous exchange of the aqueous phase for a concentrated solution of the solute in buffer. The concentration of solute in the aqueous phase was determined from the fraction of subphase volume exchanged and the concentration of the solute in the concentrated solution. From the resulting isotherms of surface pressure (π = change in surface tension) vs. bulk drug concentration, the dissociation constant and maximal surface pressure change parameters were calculated by fitting the data to the Langmuir adsorption isotherm.¹⁹ Applying the same experimental protocol without a lipid monolayer also yielded a surface pressure-concentration isotherm for each compound. These data were analyzed using the Szyszkowski equation, a combined integral form of the Gibbs and Langmuir adsorption isotherms.²⁰

MEASUREMENT OF ERYTHROCYTE PLASMA MEMBRANE LEAKAGE Bovine blood was collected immediately after slaughter. Immediately following collection, EDTA was added to the blood to achieve a final concentration of 2.7mM. The blood was cooled to 4 °C and erythrocytes were prepared as described elsewhere.²¹ A 10-ml aliquot of blood was centrifuged at $3000 \times g$ for 10 min at 4 °C, the buffy coat removed by aspiration, and the supernatant discarded. The erythrocyte pellet was suspended in 10ml 150mM NaCl and centrifuged as before and the supernatant discarded. This procedure was twice repeated with a suspension medium containing 2.7mM KCl, 1.5mM KH₂PO₄, 139mM NaCl, and 16mM Na₂HPO₄, adjusted to pH 7.4 (PBS). Approximately 4ml washed erythrocytes were resuspended in PBS to a total volume

of 10ml and stored at 4 °C. For determination of lipid phosphorous, 1.0ml erythrocyte suspension was solvent-extracted as described by Kates²² and the phosphorous content of the extract determined.²³ The concentration of phospholipid in the cell suspensions used was 1.5–3.0mM.

Erythrocyte lysis (hemolysis, i.e., hemoglobin release) was measured spectrophotometrically.²¹ Solutions of each test compound near its solubility limit were prepared in PBS and the pH was re-adjusted to 7.4 with a concentrated solution of NaOH. The suspension was centrifuged to pellet the excess compound and the supernatant was decanted. The concentration of compound in dilutions of the supernatant was determined spectrophotometrically at 299nm using calibration standard solutions of the compound prepared with lower concentrations. Ten (10) µl erythrocyte suspension (9nmol lipid phosphorous) were added to 1.5ml test solution at 37 °C. Following 30min of incubation at 37 °C, each sample was centrifuged at 4000 × g for 15min at 15 °C, the cell-free supernatant decanted, and its optical density at 577nm determined. Control experiments were performed with PBS buffer at pH 7.4 to measure erythrocyte stability and with Triton X-100 at 0.1 vol% in PBS at pH 7.4 to obtain complete hemolysis. The percentage hemolysis was calculated as 100 times the optical density of the sample supernatant at 577nm divided by that for the control treated with Triton X-100.

Erythrocyte ghosts loaded with CF (CF-ghosts) were prepared by rupturing and resealing erythrocytes in the presence of the dye.²¹ Eight (8) ml 1.2mM acetic acid, 4mM MgSO₄, pH 3.2, was added to 0.8ml erythrocyte suspension at 4 °C. After a 5-min incubation at 4 °C, the pH was adjusted to 7.4 using 1M Tris base. The sample was centrifuged at 16,000 × g for 10min at 0 °C and the pellet resuspended in 400µl 80mM CF, 20mM HEPES, 170mM NaCl, pH 7.4, at 37 °C. The ghosts were resealed by incubation for 1 hour at 37 °C, after which free CF was removed at 24 °C by passage of the sample through a column of Sephadex G-75 equilibrated with 10mM HEPES, 140mM NaCl buffer adjusted to pH 7.4. The brownish CF-ghosts eluting well ahead of the 6-carboxyfluorescein were pooled and used to measure the effects of test compounds on 6-carboxyfluorescein leakage. The quantity of membranes used in each experiment was not assessed, but was comparable to that used in hemolysis experiments.

For measurement of drug-induced 6-carboxyfluorescein leakage from CF-ghosts, 2ml of a solution of the test compound in 10mM HEPES, 140mM NaCl, pH 7.4, were added to a 1-cm cuvette and equilibrated with stirring for 3min at 37 °C. Fluorescence was recorded continuously at 510nm using an excitation wavelength of 485nm. Following the addition of 50µl CF-ghosts and the recording of fluorescence emission intensity for an additional 30min, 20µl of a 10% Triton X-100 solution (vol/vol) were added and the emission intensity was recorded for ~5min. Zero CF leakage was defined as the fluorescence intensity obtained immediately after the addition of CF-ghosts to the cuvette in the absence of drug. Complete CF leakage was defined for each sample as the value obtained after the addition of Triton X-100, less the fluorescence intensity representing zero leakage. CF leakage

for each experimental sample and control was calculated as the fluorescence intensity at 30 min after CF-ghost addition, less the zero CF leakage value intensity. Percent CF leakage was defined as the net sample fluorescence as described above divided by the net fluorescence intensity for complete CF leakage, multiplied by 100.

HISTAMINE RELEASE FROM MONODISPERSED HUMAN CONJUNCTIVAL MAST CELLS Methods detailing the preparation of monodispersed human conjunctival mast cells (HCTMC) and the assessment of HCTMC mediator release were performed as described by Yanni et al.⁹ Briefly, human conjunctival tissue mast cells were isolated from post-mortem donor tissue obtained within eight hours of death by various eye banks and transported in OPTISOL[®] corneal preservation medium. Tissues were enzymatically digested by repeated exposure (30 min at 37 °C) to collagenase and hyaluronidase (2 times with 200 U each/g tissue, followed by 2–4 times with 2000 U each/g tissue) in Tyrode's buffer containing 0.1% gelatin (TGCM). Each digestion mixture was filtered over NITEX[®] cloth (100µm mesh) and washed with an equal volume of buffer. Filtrates were centrifuged at 825 × g (7 min). Pellets were resuspended in buffer, then combined for enrichment over a 1.058 g/l PERCOLL[®] cushion. The mast cell-enriched pellet was washed and resuspended in supplemented RPMI 1640 medium for equilibration at 37 °C.

Cells were harvested from the culture plate and counted for viability (trypan blue exclusion) and mast cell number (toluidine blue O). Mast cells (5000 cells/1.0 ml total volume) were challenged at 37 °C for 15 min with goat-anti-human IgE (10 µg/ml) following treatment with test compound or TGCM buffer. Total, spontaneous, and non-specific histamine release were assessed upon exposure of mast cells to 0.1% Triton X-100, buffer only, and goat IgG (10 µg/ml), respectively. The reaction was terminated by centrifugation (500 × g, 4 °C, 10 min). Supernatants were stored at –20 °C until analyzed for histamine content by RIA. Test drugs were freshly prepared in TGCM buffer and diluted over a concentration range of 100 nM to 10 mM. Appropriate aliquots of these stock solutions were added to the culture dishes to provide the desired final concentrations.

LDH RELEASE FROM MONODISPERSED HUMAN CONJUNCTIVAL MAST CELLS Monodispersed human conjunctival mast cells were prepared as described above. Cells were counted and placed in assay tubes at a concentration of 50,000 cells per tube. Test compounds were added to the assay tubes at marketed concentrations in TGCM buffer. The total assay volume was 1.0 ml. Following a 30-min incubation at 37 °C, the cells were sedimented by centrifugation at 4 °C (500 × g for 10 min) and the supernatants were collected for the assay of histamine and LDH content. LDH was assayed spectrophotometrically at 490 nm per package insert from a 50-µl supernatant aliquot with a CYTOX 96[®] kit. Histamine was quantified by RIA as mentioned above. Separate control assays, conducted to evaluate drug effects in the LDH and histamine determination, revealed no test compound interference.

LDH RELEASE FROM CULTURED PRIMARY HUMAN CORNEAL EPITHELIAL CELLS Human corneas were obtained from the Lions Medical Eye Bank (Norfolk, VA, USA). Corneas were harvested within 12 hours post mortem and placed into OPTISOL® for shipment. Upon arrival, corneal tissue was placed with the epithelial side down into a culture dish containing 5.0ml KGM medium that was supplemented with 6 units/ml of dispase II. After two hours of incubation at 37 °C, the epithelial layer was scraped with a scalpel blade and the adherent cell scraping was transferred along with dispase solution into a centrifuge tube. Cells were sedimented by centrifugation (5 min at 130 × g). The cell pellet was resuspended in 5.0ml KGM medium and appropriate aliquots were transferred into 25cm² flasks coated with FNC. The flasks were placed in a 37 °C incubator and media were replaced after 24 hours and then every 3–4 days thereafter. For passage, cells were washed twice with 10.0ml PBS buffer and then treated with dispase II (24 units/ml) for 3 min at 37 °C. The dispase was removed and the cells were incubated for an additional 5–7 min until rounded. Cells (2×10^4) were then plated in 48-well Collagen I Cellware and grown in KGM medium until confluent.

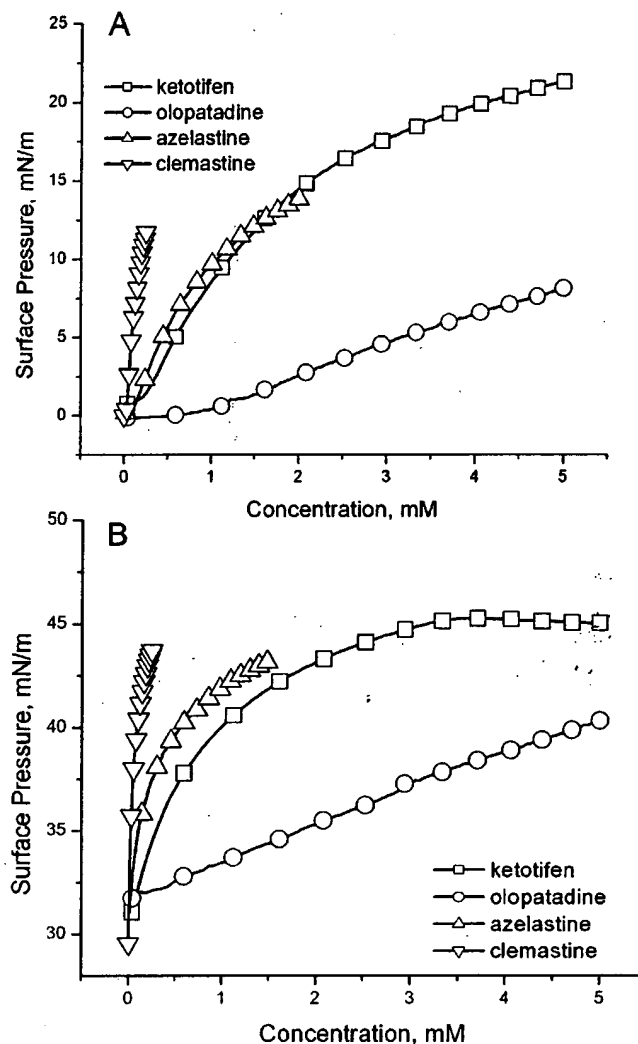
The KGM medium was removed and the cells were washed twice with 1.0ml Tyrode's buffer containing 0.1% gelatin. Test compounds were dissolved at marketed concentrations in the gelatin-containing Tyrode's buffer. An aliquot (0.5 ml) of the drug-containing buffer was added to the culture dish and cells were incubated for 30 min at 37 °C. Supernatants were then harvested and 50- μ l aliquots were assayed for LDH activity. LDH assays were conducted as described above.

Results

INTERACTION WITH THE ARGON-BUFFER INTERFACE Differences in intrinsic surface activities of olopatadine and antihistaminic agents were first assessed by measuring their propensities to increase the surface pressure of an argon-buffer interface.¹⁸ The protocol involved exchanging drug solution for buffer subphase while maintaining surface area and subphase volume constant. This was achieved by infusing a drug-containing buffer solution into the rapidly stirred subphase while simultaneously withdrawing subphase at the same rate. With available equipment, the maximum concentration of drug achieved in the subphase was limited to $\sim 1/2$ that of the infused stock solution. For representative drugs, values of the surface pressure, π , as a function of test compound concentration are shown in Figure 1A. Continuous exchange of buffer subphase with drug-containing buffer solution resulted in a concentration-dependent increase in surface pressure. For all compounds tested, the increase in surface pressure as a function of concentration exhibited an order of clemastine \approx desloratadine > azelastine \approx ketotifen > pyrilamine \approx diphenhydramine > olopatadine > emedastine = epinastine.

To more quantitatively capture intrinsic differences in surface activity among the different agents, surface pressure/drug concentration data were analyzed utilizing the Szyszkowski equation.²⁰ This equation represents the integrated form of the Gibbs equation and the Langmuir

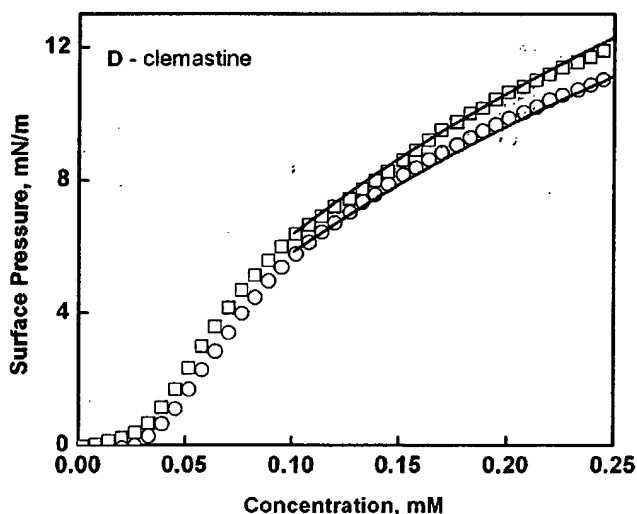
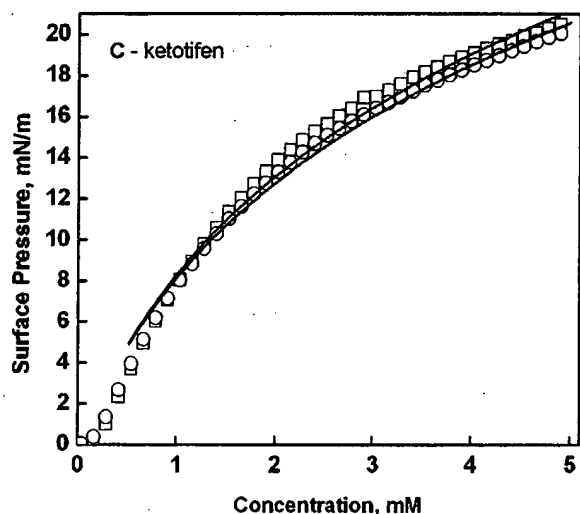
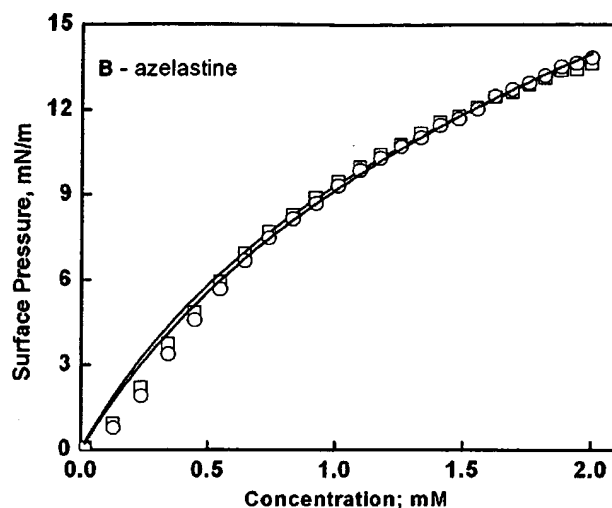
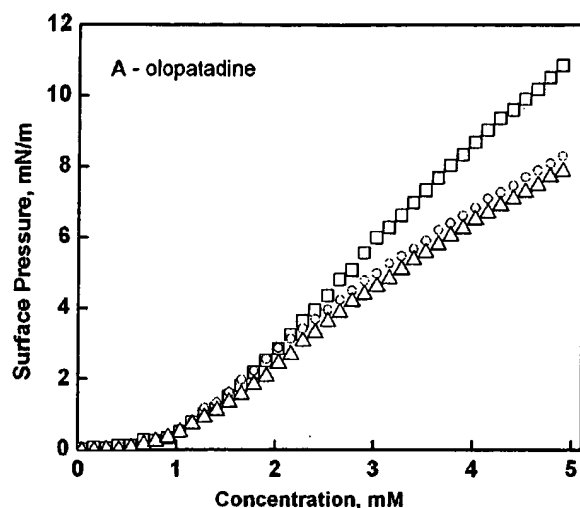
Fig. 1. Effect of test compounds on the surface pressure of (A) the argon-water interface and (B) SOPC monolayers adjusted initially at 30 ± 2 mN/m as illustrated for ketotifen, olopatadine, azelastine, and clemastine. Subphase buffer (24.4 mL) was exchanged with test compounds in buffer at a fractional rate of 0.0164/min.



adsorption isotherm and allows for an estimation of the dissociation constant ($K_{d,s}$) for drug interaction with the lipid-free interface.

Each isotherm was computer-fitted to the Szyszkowski equation using a non-linear least squares procedure. Representative data sets obtained, and their respective fits (solid lines), are shown in Figure 2. Data which showed sigmoidal behavior at low concentrations were excluded from the calculation. Similar sigmoidal behavior has been reported by Fischer et al.²⁴ for a variety of drugs adsorbing to the air-water interface. This behavior was attributed to adsorption of test compound on the walls of the Teflon trough. However, it is also a consequence of the monolayer becoming a single, liquid phase at the end of the gas-liquid phase transition. This typically occurs at approximately $\sim 1/2$ the maximum achievable surface concentration of drug.

Estimated values of the dissociation constants ($K_{d,s}$) for each compound are given in Table 1. Reliable $K_{d,s}$ estimates were obtained for most of the compounds tested. Exceptions were olopatadine and pheniramine, for which computed $K_{d,s}$ values were well above either the highest achievable drug concentration or highest concentration tested



Test compound	SOPC monolayer K_{dL} -Langmuir (mM) Mean \pm range (n = 2)	Argon-buffer interface K_{dS} -Szyszkowski (mM) Mean \pm range (n = 2)
Diphenhydramine	1.9 \pm 0.5	2.4 \pm 0.2
Pheniramine	13.1 \pm 1.1	>25 ^b
Ketotifen	0.5 \pm 0.1	0.94 \pm 0.01
Emedastine	>25 ^b	12.2 \pm 0.5
Olopatadine	>5 ^a	>5 ^a
Azelastine	0.2 \pm 0.01	0.63 \pm 0.08
Pyrilamine	4.4 \pm 0.2	6.25
Clemastine	0.05 \pm 0.01	0.15 \pm 0.01
Epinastine	1.05 \pm 0.05	17.7
Desloratadine	0.04 \pm 0.01	0.06 \pm 0.01

^aParameter could not reliably be estimated due to instrumental limitations.

^bHighest drug concentration tested = 25 mM.

Fig. 2. Surface activities of test compounds at the argon-water interface as illustrated for (A) olopatadine, (B) azelastine, (C) ketotifen, and (D) clemastine. The plots shown represent data from Figure 1A that was fitted to the Szyszkowski model over the range indicated by the solid line. Data sets obtained for olopatadine were unsuitable for fitting (see text).

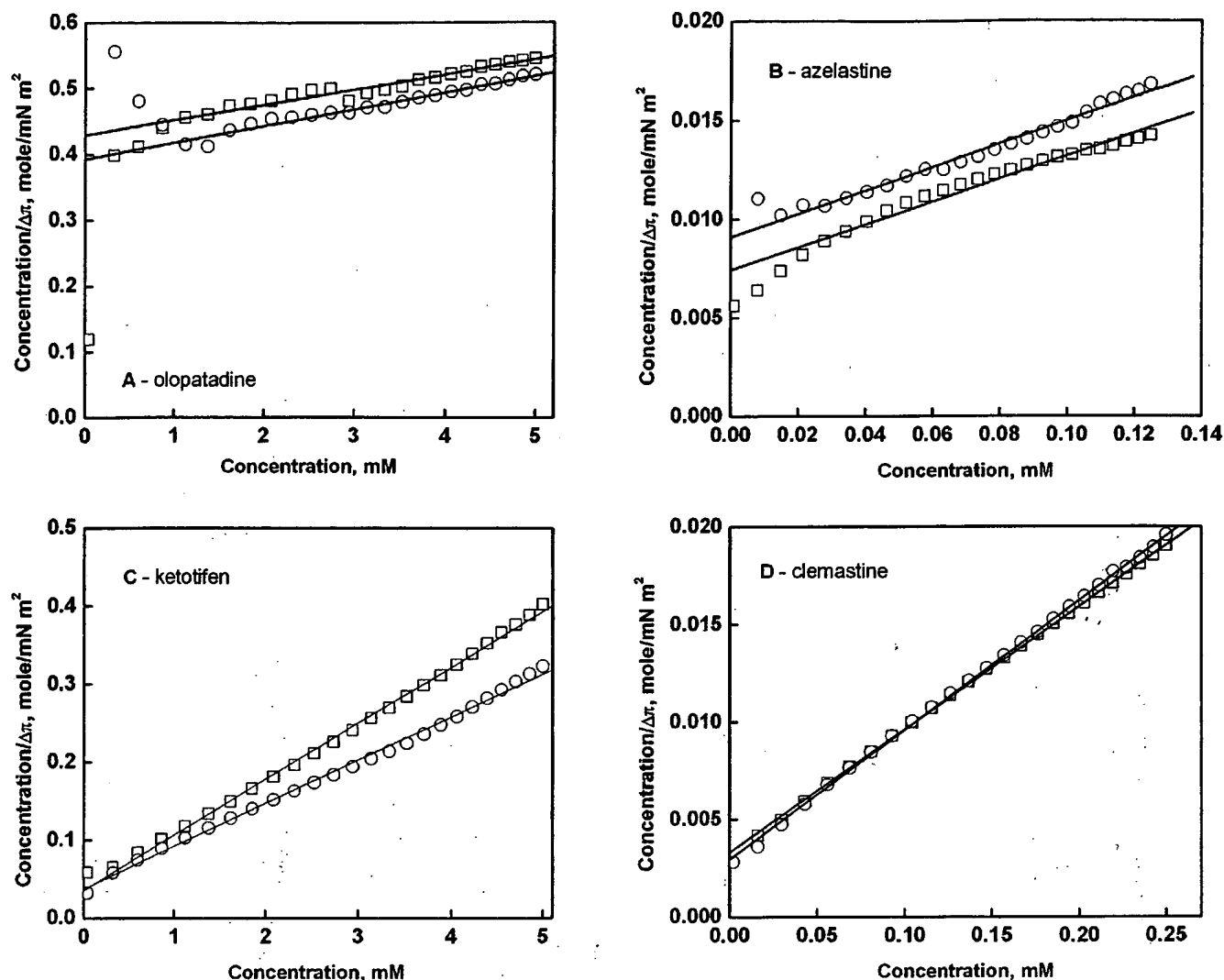
TABLE I. Parameters for adsorption of olopatadine and selected classical antihistamines to the argon-buffer interface and SOPC membranes.

(i.e., 5 mM and 25 mM, respectively). Fits for pyrilamine were borderline-acceptable in that the $K_{d,s}$ values obtained were near the highest experimental drug concentration achieved. For reliable curve fits, $K_{d,s}$ estimates of affinity for the argon-buffer interface showed a wide range, varying from 0.06 to 17.7 mM. Based on these estimates, the subset of these antihistaminic agents for which surface activity could be quantitatively analyzed can now be ranked according to their $K_{d,s}$. The order of apparent surface activity at a subphase pH of 7.5 is desloratadine > clemastine > azelastine \approx ketotifen > diphenhydramine > pyrilamine > emedastine > epinastine.

INTERACTION WITH SOPC MODEL MEMBRANES With a considerable range in intrinsic surface activity among the compounds examined, it was of interest to further explore potential differences in interaction with an interface consisting of an SOPC monolayer at a physiologically relevant packing density. Surface activity toward SOPC monolayers may be driven by specific interactions, i.e., non-ideal mixing, as well as simply amphipathicity.

Changes in surface activity as a function of drug concentration were determined by the same protocol described above, but with the interface occupied by a monolayer of SOPC at an initial surface pressure of 28–32 mN/m. This surface pressure range is believed to occur at a lipid-packing density closely resembling that encountered in cellular membranes.^{25,26} As shown with selected data sets in Figure 1B, all compounds caused a continuous increase in the surface pressure compared to the negligible change observed in sham experiments with buffer exchange alone (not shown). This demonstrates that all are sufficiently surface-active to bind to the SOPC monolayer at a physiologically relevant surface pressure. At higher concentrations, some of the antihistamines showed a tendency toward saturation behavior, e.g., ketotifen, whereas others, e.g., olopatadine, showed an essentially linear response over the experimentally accessible range of concentrations.

For analyzing the surface pressure-concentration data collected with SOPC monolayers (Fig. 1B), it is assumed that the surface concentration of the solute in the SOPC monolayer is proportional to the increase in surface pressure recorded during solute addition.²⁷ An additional requirement is that the monolayer remains in the same physical state, i.e., the liquid-expanded state. Because at 24 °C SOPC undergoes no transitions from the liquid-expanded to more condensed states before the monolayer collapses at 45–47 mN/m,²⁸ this criterion is fulfilled. Under these conditions, the Langmuir adsorption model can be applied to the surface pressure-solute concentration data.^{29,30} Figure 3 shows examples of surface pressure-concentration data (Fig. 1B) plotted according to the Langmuir model. A deviation from linearity was observed with nearly all agents tested. This deviation appears to be due to the data transformation from volume of aqueous phase exchanged to concentration and occurred only in the region that involved small changes in surface pressure at low drug concentrations. Each data set was fitted by linear regression as shown by the solid line (Fig. 3). Data that deviated from linearity at low concentrations were not included in the regression analysis. From each line, the dissociation

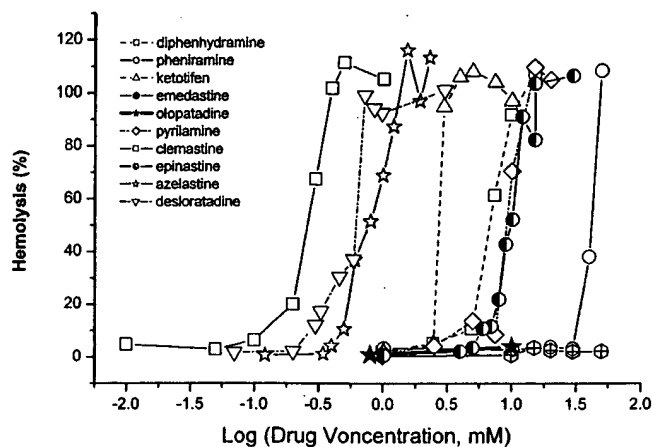


constant estimated according to the Langmuir adsorption model, $K_{d,L}$, for interaction of the drug with the SOPC monolayer was calculated. Estimates of $K_{d,L}$ values for the various antihistamines are given in Table 1. Values for interaction with the SOPC model membrane ranged from 0.04 to 13.1 mM, with desloratadine having the highest and pheniramine the lowest affinity for SOPC interaction. Similar estimates could not be made for emedastine and olopatadine because of the same experimental/solubility restrictions noted above for the application of the Szyszkowski equation. Interestingly, epinastine, which exhibited a low affinity for the argon-buffer interface ($K_{d,S} = 17.7$ mM; Table 1), demonstrated a 17-fold affinity increase for interaction with the SOPC monolayer ($K_{d,L} = 1.05$ mM) compared to the 1- to 3-fold changes calculated for the other compounds.

Overall, analysis of lipid-specific surface activity shows, like intrinsic surface activity, that all of the tested antihistaminic agents were surface-active toward monolayers of SOPC initially at 30 ± 2 mN/m. Consequently, they were expected to interact with biological membranes to

Fig. 3. Test of the Langmuir model for the adsorption of test compounds to SOPC monolayers as illustrated for (A) olopatadine, (B) azelastine, (C) ketotifen, and (D) clemastine. The plots shown represent data from Figure 1B that was plotted according to a linear transformation of the Langmuir adsorption model.

Fig. 4. Concentration-dependent effects of olopatadine and antihistamines on bovine erythrocyte hemolysis in vitro.



an extent that was influenced by a number of factors including intrinsic surface activity ($K_{d,s}$), membrane affinity ($K_{d,L}$), and solubility.

INTERACTION WITH BIOLOGICAL MEMBRANES To compare the ability of olopatadine and antihistamines to interact with natural membranes, their effects on the permeability of erythrocyte membranes were measured using two techniques. The first measured the ability of the test compounds to cause leakage of hemoglobin (MW = 64,500) from intact bovine erythrocytes, whereas the second measured the leakage of the small molecular weight tracer, 6-carboxyfluorescein (CF)(MW = 376), from CF-preloaded erythrocyte ghosts. In both cases, intact erythrocytes or erythrocyte ghosts were incubated with a solution of test compound at 37 °C for 30 min after which the percentage of solute leakage was quantified (see Methods). With the exception of emedastine, all compounds tested were evaluated over a range of concentrations that approached/included the solubility limit, unless hemolysis or CF-leakage was complete at lower concentrations. Figure 4 summarizes the effects of the test compounds on erythrocyte hemolysis.

When the cells were incubated in buffer alone, hemolysis was ~1% (not shown). However, when erythrocytes were exposed to increasing concentrations of the different test agents, olopatadine and emedastine were the only compounds that did not induce hemolysis. For agents that caused complete hemolysis, estimates were made to define the concentration that caused 50% erythrocyte hemoglobin release (H_{50}). The values obtained are given in Table 2 and varied from 0.38 to 40.7 mM, a 107-fold range. It was noted during the experiments that at concentrations of test compounds just below the onset of hemolysis, the cells became 'fragile' in that the centrifugation process caused them to rupture in the bottom of the tube. On the basis of H_{50} values, clemastine emerged as the most lytic agent. Clemastine was followed in lytic activity in the order of desloratadine = azelastine > ketotifen > diphenhydramine = pyrilamine = epinastine > pheniramine.

Similar concentration-dependent leakage was observed for those compounds tested by monitoring CF release from CF-preloaded erythrocyte ghosts (Fig. 5). While there was only a small amount of CF

Test compound	H_{50}^* (mM)	F_{50}^* (mM)
Diphenhydramine	6.6	1.3
Pheniramine	40.7	30.0
Ketotifen	2.7	1.4
Emedastine	—	14.5
Olopatadine	—	—
Azelastine	0.79	0.12
Pyrilamine	7.9	3.3
Clemastine	0.38	0.06
Epinastine	10.0	— ^a
Desloratadine	0.63	— ^a

*Interpolated/extrapolated concentration of test compound corresponding to 50% hemolysis (H_{50}) or carboxyfluorescein leakage (F_{50}) from Figures 4 and 5.

^aNot determined.

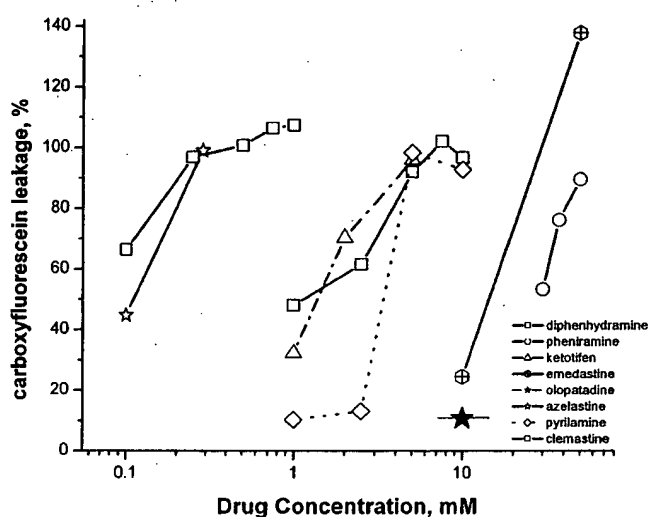


TABLE 2. Concentrations of antihistamine promoting half-maximal hemoglobin (H_{50}) and carboxy-fluorescein (F_{50}) release from intact bovine erythrocytes and erythrocyte ghosts.

Fig. 5. Concentration-dependent effects of olopatadine and antihistamines on the leakage of 6-carboxyfluorescein from bovine erythrocyte ghosts.

leakage (2.3%) in buffer-treated erythrocyte ghosts (not shown), complete release of CF was apparent when erythrocyte ghosts were exposed to the highest test doses of clemastine, azelastine, pheniramine, pyrilamine, ketotifen, diphenhydramine, and emedastine. From the data in Figure 5, concentrations that induced 50% release of CF (F_{50}) were estimated by interpolation or extrapolation. The estimated concentrations for half-maximal CF release (F_{50}) are listed in Table 2. F_{50} estimates indicate a 500-fold range (0.06–30 mM) in potencies for CF release among the different antihistaminic agents tested. The order of potency is similar to that observed for hemoglobin release from intact erythrocytes (i.e., clemastine \approx azelastine $>$ ketotifen \approx diphenhydramine $>$ pyrilamine $>$ emedastine $>$ pheniramine). Notably, olopatadine was the only agent tested that did not promote CF release from CF-loaded erythrocyte ghosts.

HISTAMINE/LDH RELEASE FROM MONODISPERSED CONJUNCTIVAL MAST CELLS The finding that olopatadine is the only topical

ocular antiallergic, antihistaminic agent that does not promote erythrocyte membrane perturbation and release of intracellular markers (Figs. 4, 5) warranted a broader re-examination of the effects of antihistamines on histamine release from human conjunctival mast cells. Previous studies evaluating the dose-dependent effect of ketotifen on histamine release from IgE-stimulated conjunctival mast cells revealed a biphasic response.⁹ The drug response consisted of an initial concentration-dependent suppression that was followed by a sudden reversal of the inhibitory effect resulting in a potentiation of histamine release. This response was in contrast to that observed with olopatadine, which promoted a concentration-dependent suppression of histamine release only, causing plateau inhibitions ($\geq 95\%$) at concentrations above 1 mM.⁷

To eliminate a stimulus-associated membrane response as a possible mechanism of the drug-induced potentiation of mast cell histamine release, the concentration-dependent effect of ketotifen and selected other, membrane perturbing ophthalmic antihistamines on the release of histamine from unstimulated (control) and anti-IgE stimulated human conjunctival mast cells was evaluated. As shown in Figure 6, increasing concentrations of ketotifen, azelastine, clemastine, and epinastine caused an initial, nearly complete suppression of histamine release from immunologically challenged mast cells. However, when drug concentrations were increased beyond these threshold doses, there was a reversal in the suppression that was followed by an enhancement of histamine release. In non-immunologically challenged (control) cells, antihistamines exhibited little effect on histamine release until achievement of threshold concentrations that coincided with the reversal of the inhibitory effects of α IgE-stimulated cells and the onset of drug-induced release of histamine (Fig. 6A,B). The concentration threshold required to induce the second-phase antihistamine response was identical for every agent tested in both control and immunologically stimulated cells.

A separate assessment was conducted that evaluated lactate dehydrogenase (LDH; 140,000 daltons) release from monodispersed, unstimulated conjunctival mast cells following a 30-min exposure to marketed concentrations of either azelastine (0.05% or 1.2 mM), epinastine (0.05% or 1.8 mM), ketotifen (0.025% or 0.6 mM), or olopatadine (0.10% or 2.7 mM). As shown in Figure 7A, azelastine promoted a nearly 9-fold increase in LDH release relative to that observed spontaneously in untreated cells. Ketotifen and epinastine were less lytic, releasing only 1/3 the quantity of LDH released by azelastine. Notably, olopatadine was devoid of lytic activity as demonstrated by release of LDH levels that were identical to those of untreated control cells.

Combined, these results indicate that, with the exception of olopatadine, all ophthalmic antihistamines at low, millimolar concentrations perturb cell membranes, leading to cell lysis and release of both small and large molecular weight intracellular constituents. Moreover, the data show that drug-induced histamine release from human conjunctival mast cells appears to be caused by a simple drug/membrane interaction rather than events associated with cell stimulation.

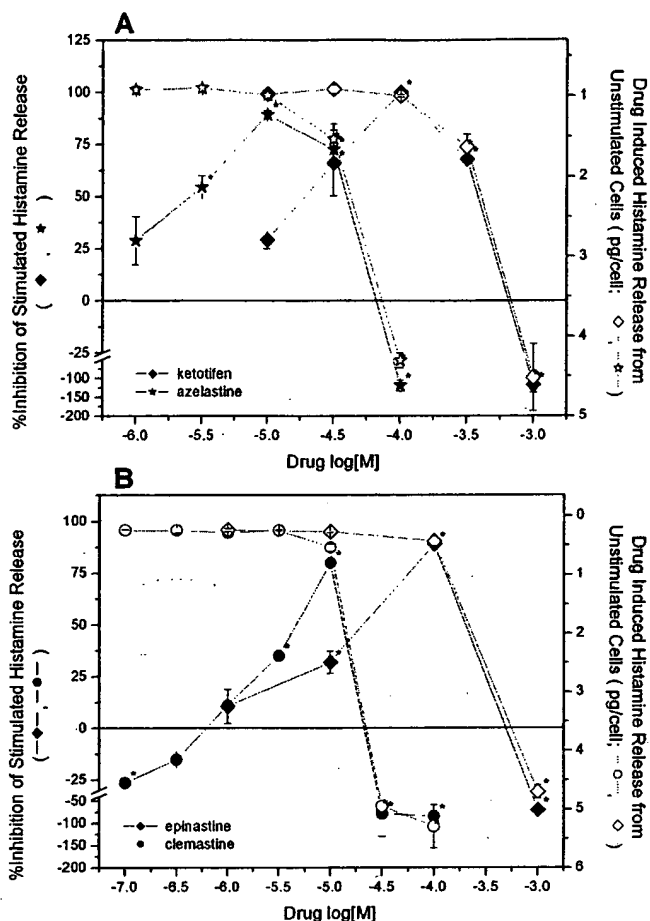
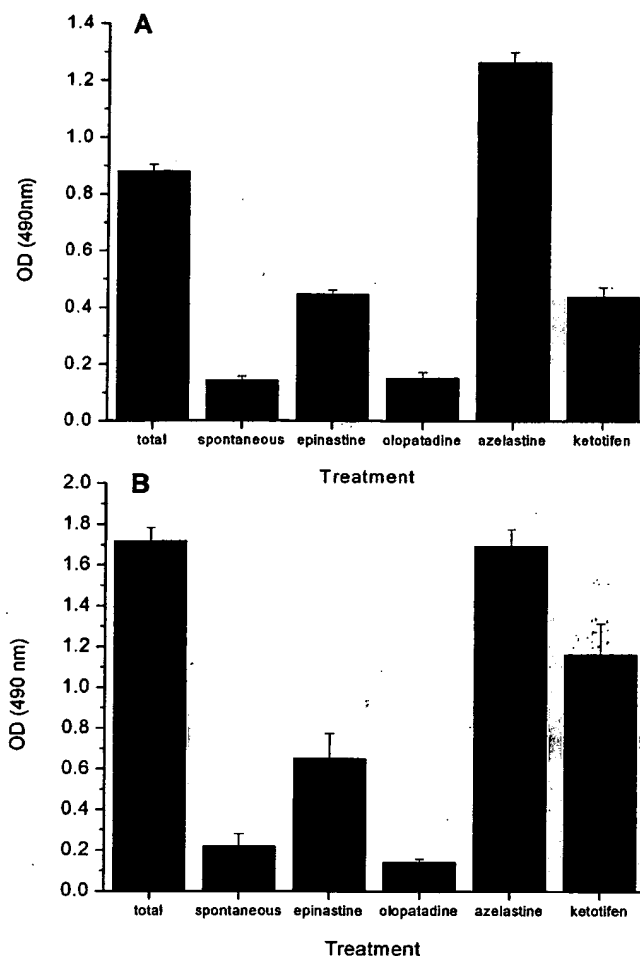


Fig. 6. Effects of (A) ketotifen and azelastine and (B) epinastine and clemastine on histamine release from control (unstimulated) and immunologically (α IgE) stimulated preparations of monodispersed human conjunctival mast cells.

LDH RELEASE FROM PRIMARY CULTURES OF HUMAN CORNEAL EPITHELIAL CELLS The finding that short-term exposure (30 min) of monodispersed human conjunctival mast cells to marketed concentrations of azelastine, epinastine, and ketotifen resulted in both histamine and LDH release raised the possibility of cytotoxic effects to ocular surface epithelial cells. To assess this possibility, human corneal epithelial cells were isolated from cadaver tissue and grown to confluence in primary cultures (see Methods). Exposure of these cells to marketed concentrations of azelastine, epinastine, ketotifen, and olopatadine for a period of 30 min demonstrated findings (Fig. 7B) virtually identical to those observed with monodispersed human conjunctival mast cells (Fig. 7A). Azelastine caused a maximal LDH release similar to that achieved with a 30-min treatment with the detergent, Triton-X100, used to determine total cellular LDH content. Ketotifen and epinastine exhibited reduced cytotoxic effects, releasing 70% and 38% of the total intracellular LDH content, respectively. Olopatadine, on the other hand, exhibited no cytotoxicity, with extracellular LDH levels not exceeding those of vehicle-treated control cells (i.e., spontaneous release; Fig. 7B).

Discussion In this report, we describe the intrinsic surface activity and interaction with model and natural membranes of olopatadine, a

Fig. 7. Lactate dehydrogenase (LDH) release from preparations of (A) monodispersed human conjunctival mast cells and (B) primary cultures of human corneal epithelial cells following a 30-min exposure to marketed concentrations of epinastine (0.05% or 1.8mM), olopatadine (0.1% or 2.7mM), azelastine (0.05% or 1.2mM), and ketotifen (0.025% or 0.6mM).



unique topical ocular antiallergy agent with antihistamine action and mast cell-stabilizing properties. These physical characteristics are compared to those of selected classical antihistamines and other topical ocular antiallergic drugs.

To assess specificity to interact with model membranes, two procedures were employed. The first method measured the affinity of the antihistaminic agents to interact with a lipid-free argon-buffer interface, thus permitting characterization of their intrinsic surface activity. The second procedure measured their interaction with an SOPC monolayer, thereby allowing the determination of their affinity for an uncharged phospholipid membrane interface. Both methods have been used previously with antihistamines,^{15,16} and it was confirmed in the present study that these agents are surface-active (Fig. 1A).

In general, the continuous buffer exchange protocol used with both methods allowed for the determination of surface activity of the compounds with minimal perturbation of the monolayer compared to the stepwise solvent-based injection procedure used previously by others.²⁴ The principal advantage of this method is that it can be used to attain drug concentrations under lipid monolayers that approach the solubility limits of the compounds being tested. The limitations of the exchange procedure as currently implemented are that the concentra-

tion of the test compound in the aqueous phase cannot be increased beyond half the concentration of the stock solution of the test compound. While this procedure has the advantage of monolayer surface activity measurement with minimal perturbation, it requires greater quantities of test material than would be needed with stepwise injections.

Two of the compounds in this study, diphenhydramine and pheniramine, have been characterized before with respect to their surface activities, in both the presence and absence of a phosphatidylcholine monolayer.^{15,16} The monolayers used in the earlier experiments were composed of 1,2-dipalmitoyl-glycero-*sn*-3-phosphocholine rather than SOPC; the subphase was water without pH control rather than buffer; and the constant area at which the data were reported was 100 Å²/molecule rather than the 76.5 ($\pi = 30$ mN/m) value in the present study. The differences in the data are consistent with the differences in experimental conditions, particularly the molecular area of the phospholipid. Specifically, the changes in surface pressure induced by a 5 mM concentration of diphenhydramine or pheniramine were comparable but smaller, 12.7 and 2.6 mN/m, than the values reported earlier, 16.5 and 2.8 mN/m.¹⁵ Data obtained earlier in the absence of a lipid monolayer¹⁶ are reported as the concentration of compound required to give a surface pressure of 10 mN/m. Those values are 3.0 and 35 mM for diphenhydramine and pheniramine, respectively, a difference of about 11-fold. In the present study, the values 3.4 and 19.5 differ by 6-fold, but the trend is the same.

All of the compounds studied exhibited surface activity using both protocols and dissociation constants could be determined for most (Table 1). Where comparison of dissociation constants was possible, the $K_{d,L}$ values determined with the SOPC monolayer using the Langmuir adsorption equation were lower than those determined by the Szyszkowski equation. The lower values obtained with SOPC imply that there is a contribution to the energy of interaction with SOPC which exceeds the work necessary to penetrate the monolayer. For most of the compounds, the ratio $K_{d,S}/K_{d,L}$ ranged from 1.3 to 3.2. The exception was epinastine, for which the ratio was 16.8. Excluding epinastine, the relationship between the constants $K_{d,S}$ and $K_{d,L}$ (Table 1) revealed a linear correlation ($R^2 = 0.99$; slope = 0.71) with an intercept near zero (not shown). This linear correlation indicates the relative equivalence of the two methods in the present case. It also suggests that aside from epinastine, there are no antihistamine-specific interactions with SOPC that drive the antihistamine binding to the interface. The apparent lack of specificity may be a consequence of the positive charge of all of the test compounds at pH 7.4 and the lack of charge of the model membranes. Such a situation would also be encountered in natural membranes that have an outer leaflet composed of phospholipids, mostly phosphatidylcholine and sphingomyelin.³¹ If the model membrane had contained diacylphosphatidylserine or other charged amphiphiles²⁴ or if the test compounds had differed in charge,²⁹ such consistency would be unlikely.

Notably, olopatadine and emedastine, although surface-active, were insufficiently so to permit an assessment of their dissociation constants

for interactions with SOPC using the Langmuir model. This restriction was due to a limit in compound solubility (olopatadine) and insufficient intrinsic surface activity (emedastine).

Three complementary in-vitro models were used to probe for functional consequences of interactions of the surface-active agents with biological membranes. These models, in contrast to the surface activity and membrane affinity measurements, were not limited to test concentrations of half that of the solubility limit of the test drug, thus permitting the evaluation of olopatadine test concentrations that were near the solubility limit. The initial model utilized both intact bovine erythrocytes and erythrocyte ghosts to measure membrane leakage of both large and small molecular weight components. Another model utilized human conjunctival mast cells and evaluated histamine release from intracellular granules and lactate dehydrogenase (LDH) from the cytoplasmic compartment. The third model measured LDH release from primary cultures of human corneal epithelial cells. The testing in the latter model was restricted to marketed drugs and their corresponding concentrations only because of its relevance to topical ocular drug exposure in the clinical setting.

Using concentrations of test compounds up to their solubility limits, olopatadine and emedastine were the only agents that did not promote membrane lysis of intact erythrocytes as indexed by hemoglobin release. While emedastine was able to restrict membrane perturbation and the release of hemoglobin from intact erythrocytes, it promoted sufficient perturbation of the more fragile membranes of erythrocyte ghosts, leading to the release of the small molecular weight marker, 6-CF, that was contained within the ghosts. Olopatadine was the only agent that did not affect the permeability of membranes, even those of the more fragile erythrocyte ghosts. This lack of perturbation was observed up to the solubility limit of the drug (~10mM).

The drug concentrations at which leakage was 50%, H_{50} and F_{50} , are compared in Table 2. The data indicate that F_{50} values were lower than H_{50} in all cases. These results were reasonable because CF is a much smaller molecule than hemoglobin. Notably, there is a high degree of correlation ($r = 0.96$) between the affinity of compound binding (K_{dL}) to an SOPC monolayer and drug concentration causing half-maximal release of hemoglobin (H_{50}) or carboxyfluorescein (F_{50}) from intact bovine erythrocytes or erythrocyte ghosts, respectively (Fig. 8A). Moreover, the slope of near unity (slope = 0.76) further supports the conclusion that erythrocyte membrane perturbation and intracellular marker release are both consequences of partitioning of the amphipathic drugs into erythrocyte membranes. The linearity also argues against a receptor-based mechanism unless binding of the compounds to the receptor is also dominated by non-specific hydrophobic interactions. The direct correlation between the compound binding affinities to SOPC monolayers and the drug concentrations causing half-maximal lytic release of hemoglobin from erythrocytes is further corroborated by a direct relationship between the threshold drug concentrations required to cause the release of histamine from human conjunctival mast cells (Fig. 8B). Consequently, there is a strong correlation between drug-induced histamine release from human conjuncti-

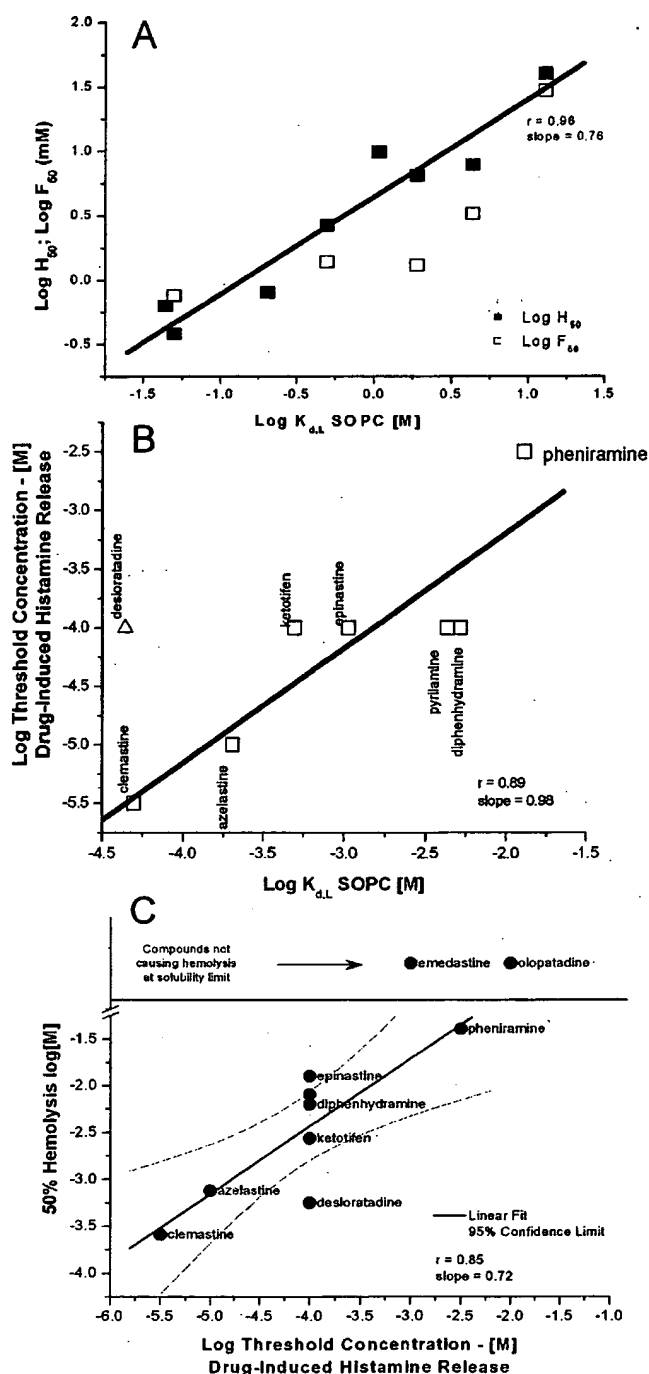


Fig. 8. (A) Relationship of test compound concentrations that promoted half-maximal hemoglobin release (H_{50}) and 6-carboxyfluorescein leakage (F_{50}) with the dissociation constants for SOPC-drug interaction. (B) Relationship of test compound threshold concentrations for drug-induced histamine release from human conjunctival mast cells with the dissociation constants for SOPC-drug interaction. (C) Relationship of test compound concentrations causing half-maximal hemoglobin release (H_{50}) with test compound threshold concentrations for drug-induced histamine release from human conjunctival mast cells.

val mast cells and drug-induced hemoglobin release from intact bovine erythrocytes (Fig. 8C).

Another indicator that erythrocyte leakage is a membrane phenomenon is the consistency of the model membrane surface pressure evaluated at H_{50} and F_{50} for each compound. These pressure values were calculated using H_{50} or F_{50} and the average parameter values for the Langmuir and Szyszkowski isotherms. Particularly, the calculations using H_{50} in the Langmuir model indicate that the calculated $\Delta\pi$ at which hemolysis is 50% has a remarkably constant value of $12.9 \pm$

1.1 mN/m. If this value is added to 30 mN/m, the initial surface pressure of the SOPC monolayer, the total predicted surface pressure is ~43 mN/m. This value lies well above the postulated internal 'surface pressure' of biological membranes of 30–35 mN/m^{25,26} and is near the monolayer collapse pressure of ~47 mN/m exhibited by the SOPC monolayer.²⁸ Although the data are more scattered, the calculated surface pressure from F_{50} values is lower, ~40 mN/m, a value still well above accepted membrane pressures. Interestingly, a correlation has also been observed between detergent-induced hemolysis and detergent-induced surface pressure changes in cholesterol monolayers initially at 4 mN/m. In this case, however, the monolayer surface pressure at which hemolysis was complete was only 34 mN/m.²⁹ The higher value in the present study suggests that charged soluble amphipaths may interact more strongly with chain-melted zwitterionic SOPC than with the relatively rigid cholesterol molecule.

Of potential clinical significance is the finding that concentrations of topical ocular antihistamine drugs contained in marketed products are capable of perturbing ocular surface cell (corneal epithelium) membranes. The data demonstrating LDH release from primary human corneal epithelial cells suggest that patient intolerance or drug-induced irritations such as stinging and burning may be the result of subtle ocular surface epithelial damage at the high concentrations applied onto the eye. Olopatadine's lack of non-specific effects on cell membrane integrity may explain the ocular comfort and compatibility observed with this agent in comparison to others.^{32,33}

Overall, this study has shown that the lytic activity of the series of cationic, classical, and ophthalmic antihistamines, excluding olopatadine, correlates well with their surface activities at model membrane interfaces, particularly SOPC monolayers. This correlation suggests that, as has been shown for other classes of compounds, cell lysis by antihistamines is not a consequence of metabolic events but of their nonspecific direct alteration of the permeability properties of the plasma membrane. Like the antihistamines studied, olopatadine is also surface-active and partitions into model and biological membranes. Its unique inability to cause lysis appears to be a consequence of its relatively low surface activity combined with its limited aqueous solubility. These parameters may be reflected in reduced ocular side effects and greater patient acceptance.

References

- 1 Mota I, da Silva WD. The anti-anaphylactic and histamine releasing properties of the anti-histamines: their effect on mast cells. *Br J Pharmacol.* 1960;15:396–404.
- 2 Lichtenstein LM, Gillespie E. The effects of H₁ and H₂ antihistamines on allergic histamine release and its inhibition by histamine. *J Pharmacol Exp Ther.* 1975;192:441–450.
- 3 Church MK, Gradidge CF. Inhibition of histamine release from human lung in vitro by antihistamines and related drugs. *Br J Pharmacol.* 1980;69:663–667.
- 4 Frisk-Holmberg M, Van der Kleijn E. The relationship between the lipophylic nature of tricyclic neuroleptics and anti-depressants and histamine release. *Eur J Pharmacol.* 1972;18:139–147.

- 5 Kazimierczak W, Peret M, Maslinski C. The action of local anaesthetics on histamine release. *Biochem Pharmacol.* 1976;25:1747-1750.
- 6 Seeman P. The membrane actions of anaesthetics and tranquilizers. *Pharmacol Rev.* 1972;24:583-655.
- 7 Felmeister A. Relationships between surface activity and biological activity of drugs. *J Pharm Sci.* 1972;61:151-164.
- 8 Attwood D, Florence AT. Surface activity and colloidal properties of drugs and naturally occurring substances. In: *Surfactant systems*. New York: Chapman and Hall, 1983; 124-228.
- 9 Yanni JM, Stephens DJ, Miller ST, Weimer LK, Graff G, Parnell D, Lang LS, Spellman JM, Brady MT, Gamache DA. The in vitro and in vivo ocular pharmacology of olopatadine (AL-4943A), an effective anti-allergic/antihistaminic agent. *J Ocular Pharmacol Ther.* 1996;12:389-400.
- 10 Sharif NA, Xu SX, Miller ST, Gamache DA, Yanni JM. Characterization of the ocular antiallergic and antihistaminic effects of olopatadine (AL-4943A), a novel drug for treating ocular allergic diseases. *J Pharmacol Exp Ther.* 1996;278:1252-1261.
- 11 Yanni JM, Miller ST, Gamache DA, Spellman JM, Xu S, Sharif NA. Comparative effects of topical ocular anti-allergy drugs on human conjunctival mast cells. *Ann Allergy Asthma Immunol.* 1997;79:541-545.
- 12 Abelson MB. Evaluation of olopatadine, a new ophthalmic antiallergic agent with dual activity, using the conjunctival allergen challenge model. *Ann Allergy Asthma Immunol.* 1998;81:211-218.
- 13 Ishii H, Sasaki Y, Manabe H, Ikemura T, Sato H, Ichikawa S, Shiozaki S, Kitamura S, Ohmori K. General pharmacology of KW-4679 an antiallergic drug (1st report) - Effects on central nervous system, autonomic nervous system and peripheral nervous system. *Clin Pharmacol Ther.* 1995;5:1421-1440.
- 14 Kosaka Y, Toda Y, Ishii A. Effect of KW-4679 on cyclic AMP content in rat peritoneal mast cells. Kyowa Hakko Kogyo, Ltd. Internal Report 87-142(Y) 1987.
- 15 Attwood D, Udeala OK. The interaction of antihistamines with lecithin monolayers. *J Pharm Pharmacol.* 1975;27:806-810.
- 16 Attwood D, Udeala OK. The surface activity of some antihistamines at the air-solution interface. *J Pharm Pharmacol.* 1975;27:754-758.
- 17 Seeman P, Weinstein J. Erythrocyte membrane stabilization by tranquilizers and antihistamines. *Biochem Pharmacol.* 1966;15: 1737-1752.
- 18 Brockman H, Graff G, Spellman J, Yanni J. A comparison of the effects of olopatadine and ketotifen on model membranes. *Acta Ophthalmol Scand.* 2000;78:10-15.
- 19 Adamson AW. The solid-liquid interface. Adsorption from solution. In: *Physical chemistry of surfaces*. 2nd ed. New York: Interscience Publishers, 1967; 397-435.
- 20 Chattoraj DK, Birdi KS. Adsorbed monolayers and energies of adsorption. In: *Adsorption and the Gibbs surface excess*. New York: Plenum Press, 1984; 133-177.
- 21 Keukens EAJ, De Vrije T, Jansen LAM, De Boer H, Janssen M, De Kroon AIPM, Jongen WMF, De Kruijff B. Glycoalkaloids selectively permeabilize cholesterol containing biomembranes. *Biochim Biophys Acta.* 1996;1279:243-250.
- 22 Kates M. Lipid extraction procedures. In: Work TS, Work E, editors. Vol. 3, Part II of Laboratory Techniques in Biochemistry and Molecular Biology. *Techniques of lipidology: isolation, analysis and identification of lipids*. Amsterdam: North-Holland Publishing, 1972; 347-353.
- 23 Bartlett GR. Phosphorus assay in column chromatography. *J Biol Chem.* 1959;234:466-469.
- 24 Fischer H, Gottschlich R, Seelig A. Blood-brain barrier permeation:

- molecular parameters governing passive diffusion. *J Membr Biol.* 1998;165:201-211.
- 25 Marsh D. Lateral pressure in membranes. *Biochim Biophys Acta.* 1996;1286:183-223.
 - 26 MacDonald RC. The relationship and interactions between lipid bilayers vesicles and lipid monolayers at the air/water interface. In: Rosoff M, editor. *Vesicles*. New York: Marcel Dekker, 1996; 3-48.
 - 27 Pethica BA. The thermodynamics of monolayer penetration at constant area. *Faraday Soc Trans.* 1955;51: 1402-1411.
 - 28 Cunningham BA, Tsujita T, Brockman HL. Enzymatic and physical characterization of diacylglycerol-phosphatidylcholine interactions in bilayers and monolayers. *Biochemistry.* 1989;8: 32-40.
 - 29 Pethica BA, Schulman JH. The physical chemistry of haemolysis by surface-active agents. *Biochem J.* 1953;53:177-185.
 - 30 Mayer LD, Nelsestuen GL, Brockman HL. Prothrombin association with phospholipid monolayers. *Biochemistry.* 1983;22: 316-321.
 - 31 Zwaal RFA, Roelofsens B, Comfurius P, Van Deenen LLM. Organization of phospholipids in human red cell membranes as detected by the action of various purified phospholipases. *Biochem Biophys Acta.* 1975;406:83-96.
 - 32 Berdy GJ, Spangler DL, Bensch G, Berdy SS, Brusatti RC. Comparison of the relative efficacy and clinical performance of olopatadine hydrochloride 0.1% ophthalmic solution and ketotifen fumarate 0.025% ophthalmic solution in the conjunctival antigen challenge model. *Clin Ther.* 2000;22:826-833.
 - 33 Aguilar AJ. Comparative study of clinical efficacy and tolerance in seasonal allergic conjunctivitis management with 0.1% olopatadine hydrochloride versus 0.05% ketotifen fumarate. *Acta Ophthalmol Scand.* 2000;78:57-60.

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